Will giant polar amphipods be first to fare badly in an oxygen-poor ocean? Testing hypotheses linking oxygen to body size.

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Abstract

It has been suggested that giant Antarctic marine invertebrates will be particularly vulnerable to declining O_2 levels as our ocean warms in line with current climate change predictions. Our study provides some support for this Oxygen Limitation Hypothesis with larger body sizes being generally more sensitive to O_2 reductions than smaller body sizes. However, it also suggests that the overall picture is a little more complex. We tested predictions from three different, but overlapping, O_2-related hypotheses accounting for gigantism, using four, Antarctic amphipod species encompassing a wide range of body sizes. We found a significant effect of body size, but also of species, in their respiratory responses to acutely declining O_2 tensions. The more active lifestyle of intermediate-sized *Prostebbingia brevicornis* was supported by a better respiratory performance than predicted by the Oxygen Limitation Hypothesis alone, but consistent with the Symmorphosis Hypothesis. We suggest that giant polar amphipods are likely be some of the first to fare badly in an O_2-poor ocean. However the products of past evolutionary innovation, such as respiratory pigments that enhance O_2- transport and novel gas exchange structures, in some species, may offset any respiratory disadvantages of either large or small body size.

Introduction

It is widely recognised that climate change will impact marine biodiversity in manifold and complex ways, and there will be winners and losers [1-3]. Understanding the biological mechanisms determining such outcomes is paramount, especially if those mechanisms generalize across taxa [4-6]. In particular determining the physiological features that might be associated with gigantism, and how giant aquatic animals will fare in a future oxygen (O_2)-poor ocean [7] has attracted attention [8-14]. One longstanding notion is that living in cold, O_2-rich waters may favour the evolution of more larger bodied species, compared with living in warmer (and so containing less dissolved O_2) temperate and tropical waters. DeBroyer [15] showed that about 1/3rd of Antarctic amphipods had body sizes twice that of the mean body size in their genus. However it was Chapelle and Peck [8] who first established, and subsequently strengthened [16,17], the evidence for a quantitative relationship between maximum body size and environmental O_2. They championed the idea that polar gigantism is possible because (1) more O_2 is available in cold, compared with warm, waters and (2) there is reduced metabolism (and so low rates of O_2 uptake [$\dot{V}O_2$]) at such sub-zero temperatures. Based on the strength of these relationships they formulated their 'Oxygen (Limitation) Hypothesis', i.e. maximum potential body size is limited by O_2 availability. They went on to suggest that, '(g)iant amphipods may therefore be amongst the first species to disappear if global temperatures are increased or global O_2 levels decline'. Since then their Oxygen Limitation Hypothesis has attracted attention including empirical tests of its key prediction that, all else being equal, reduced O_2 concentration should have a disproportionately large effect on the performance of large-bodied species. While tests of this prediction have provided some support (e.g. [9, 18-20], the evidence remains equivocal, with seemingly

different outcomes for animals that do or do not possess specialised respiratory gas exchange surfaces and transport mechanisms [12, 21-23] as not always is ‘all else equal’ [9].

Since then two alternative hypotheses linking O_2 to body size have been suggested. The first is that animals of different body size should show symmorphosis (*sensu* Weibel et al. [24]), i.e. their respiratory systems have been the subject of evolutionary change and, irrespective of body size, have been shaped to supply adequate O_2 for metabolism, minimizing redundant excess capacity [9]. The ‘Symmorphosis Hypothesis’ predicts that any effects of reduced O_2 should be independent of body size. If supported, this would be the death-knell of the idea of relating size to vulnerability [25]. The second is the ‘Respiratory Advantage Hypothesis’ [10]. This is predicated on the seemingly counter-intuitive idea that despite reduced metabolic demand and greater O_2 solubility in cold waters, O_2 bioavailability is lower. This is because any potential benefit of increased O_2 solubility in the cold may be negated by a concomitant reduction in the O_2 diffusion coefficient, the coefficient affected by a combination of the reduction in the thermal motion of O_2 molecules and increased viscosity at low temperatures [26]. Therefore large body size in an animal which does not have well developed mechanisms for respiratory regulation (e.g. possession of a respiratory pigment like hemoglobin/hemocyanin or well developed hyperventilation response) bestows a respiratory advantage that can overcome viscosity-related, low O_2 availability in polar waters [10]. Consequently, one prediction from the Respiratory Advantage Hypothesis is that gigantism should be absent in polar groups characterised by good respiratory control. A second prediction is that reduced O_2 should have a disproportionately large effect on the performance of small-bodied individuals.

Moran and Woods [25] identify at least eight hypotheses explaining ‘gigantism’. While it is unlikely that body size variation generally, and gigantism specifically, can be attributable to simple or univariant explanations [25, 27], the three O_2 theories outlined above are testable, and amphipod crustaceans in particular, given their relatively conservative body plan, and the fact they have already contributed much to our understanding, make excellent models. Therefore the aim of this present study was to test the predictions arising from these three hypotheses using carefully selected amphipod species. The main performance measure chosen was the ability to maintain respiratory independence during exposure to acutely declining PO_2s. It does not seem unreasonable to assume that such respiratory independence is a reasonable fitness proxy given the importance of aerobic metabolism in a relatively active group such as the amphipods.

All other measures are features that underpin that respiratory performance, with a view to determining how much ‘all else is equal’. (1)The ventilation response to acutely declining PO_2s was quantified as this underpins the respiratory performance measure, at least acutely although in more hypoxic environments it may also be a chronic response [28]. (2) Total gill area was quantified for three of the species as it is possible that larger species, if their physiology is compromised by internal hypoxia, could have developed a greater mass-specific gill area. *Prostebbingia brevicornis*, in common with some other amphipod species (e.g.[29,30]), appeared to have extrabranchial gas exchange surfaces in the form of modified coxal plates. This was the only one of the four species that displayed this modification. Therefore their coxal gas exchange area was estimated and expressed as a proportion of total gill area. (3) Finally evidence was sought for the presence of a respiratory pigment which would improve O_2

transport from the gills to the tissues, and potentially contribute to respiratory performance during hypoxia.

Studies of the activity of Antarctic marine ectotherms, to date, show that activity rates are generally low, with a few notable exceptions where adaptations, such as increased mitochondrial densities, have permitted locomotory speeds to be maintained [31]. In the majority of species, where activity is limited by the constant cold of the Southern Ocean, symmorphosis predicts that their respiratory organs will have lost the capacity to respond to the increased O_2 demand caused by elevated metabolic rates at warmer body temperatures [32]. Tests of the thermal response of locomotion can therefore be used to assess the temperature sensitivity of activity, which in turn may provide insights into the flexibility of O_2 supply [19].

Four species of widely differing body sizes were chosen (species name and recorded wet mass range from the field): *Paraceradocus miersi* (0.042 – 2.7 g), *Shraderia gracilis* (0.038 – 0.062 g) *Probulisca ovata* (0.003 – 0.001 g) and *Prostebbingia brevicornis* (0.037 – 0.103 g) (see Supplementary materials 1 for further details). The first three because they are phylogenetically-distant from one another (3 different superfamilies); all of them appear similarly sluggish at low temperatures, but they encompass a very wide range of body sizes. Access to congeneric species covering a wider range of body sizes, or investigating a large enough number of species of known phylogeny to incorporate phylogenetic non-independence, would have been ideal but neither option proved possible. The fourth species *Prostebbingia brevicornis* was chosen because, because it has a larger body size than the relatively closely-related, *S. gracilis* (same family) and its size range overlaps with two of the other species. It also appears to be considerably more active and has a different body form from the other three species.

These four species are abundant and co-occur in the same habitat, a relatively shallow water (6 - 8 m depth) boulder field close to Rothera Research Station, in coastal waters of the western Antarctic Peninsula (Supplementary materials 1). There is also some evidence that even when overlying water is normoxic small individuals living beneath those boulders may experience reduced PO_2s (Supplementary materials 1).

Materials & Methods

Animal material

Amphipods were collected close to the British Antarctic Survey research station, Rothera Point on the western Antarctic Peninsula (see Supplementary materials 1 for collection details). Upon capture amphipods were transferred to containers filled with sea water at the same temperature as their surroundings (T = 0°C) and taken to the laboratory within 60 min of capture. Here amphipods were maintained in a number of Aquarium Tank Fish Hatchery and Fry Breeder Units (All Pond Solutions, Uxbridge, UK) that floated, partially-submerged, in large aquaria supplied with untreated running sea water (T = -1°C to 0 °C, S = 35, DO = 100 - 106 % air saturation) pumped directly from South Cove adjacent to the laboratory. Each unit was supplied with squares of plastic mesh that provided shelter and greatly reduced cannibalism and agonistic interactions. All amphipods were kept unfed, for at least 48 h under these conditions before being used in any of the experiments described below.

Measurement of $\dot{V}O_2$ during acutely declining PO_2s

Rates of O_2 uptake during acutely declining PO_2s were measured (T = -1.0°C for all four species using a well-established, closed respirometer technique [33] (also see

Supplementary materials 2A). This technique was compared with another well established but semi-open technique that has been used extensively for amphipods [34]. Two of the Antarctic species examined in this current study (*P. miersi* and *P. brevicornis*) were collected the previous season and returned live from Antarctica to Plymouth where the comparison took place. There was no significant difference in $\dot{V}O_2$s between the techniques (Supplementary materials 2A).

Immediately the respirometer bottles were sealed, each containing an individual amphipod, the water was gently stirred using a magnetic stirrer (Mighty mouse IKA^R color squid)) and the O_2 concentration in the water measured. Oxygen concentration (sensitivity 0.1 %) was measured using a Presens system (Fibox 4 Precision Sensing GmbH, Regensburg, Germany). Concurrently ventilation rate was quantified visually using a hand-held magnifying glass (x 10) and stop watch. The time taken for 20 beats was measured in triplicate and an average calculated. Oxygen and ventilation measurements were made at 30 min intervals until all of the O_2 was exhausted within the bottle or until there were no visible signs of movement from the amphipod (movement of pleopods, pereopods or antennae). At this time individuals were removed, carefully blotted dry and weighed using a precision semi-microbalance (Genius ME235S, Sartorius, Bradford, MA). $\dot{V}O_2$ was calculated as µL.g wet mass^{-1}.h^{-1} but in species comparisons of the effect of acutely declining PO_2s, uptake was expressed as the % of the mean normoxic rate for that species, at the corresponding environmental PO_2 (expressed as % air saturation).

Quantifying oxyregulatory ability

To characterise the relationship between $\dot{V}O_2$ and environmental PO_2 for individuals of each species we calculated a Regulation Index (RI), a novel measure of oxyregulation proposed by Mueller and Seymour [35]. A number of techniques have

been suggested for comparing oxyregulatory ability in aquatic animals (e.g. [36-37] but with no general agreement on which is best. The RI was chosen because the measure is quantitative and more appropriate for working with species that show relatively poor oxyregulatory ability. Briefly $\dot{V}O_2$ (as % normoxic rate) was plotted as a function of environmental PO_2 for each individual, and in each case a line was fitted using a third order polynomial which best described the curve. The area enclosed by this line of best fit and a straight line describing the relationship that would be present if the individual showed absolute oxyconformity was quantified using integration (using Graph Pad, Prism 7). An RI = 1 represents perfect oxyregulation, 0 perfect oxyconforming (See Supplementary materials 2 for examples). Thus calculated values within this range are good measures of respiratory independence under conditions of acutely declining PO_2s. A mean RI value was calculated for each individual, from each species. We then tested for an effect of body mass (as a fixed factor) on RI (as response variable) controlling for species (as a random factor).

Measurement of gill and extrabranchial exchange surface areas

Gill area measurements were carried out on fresh material of *P. miesi*, (n = 6) *P. brevicornis* (n = 9) and *S. gracilis* (n = 9) (but not successfully on *P. ovata* as they were so small) following closely the method of Moore and Taylor [38]. This involved carefully excising each gill from individual amphipods, encompassing a wide range of body masses (0.016 – 3.30 g wet mass). Gills were carefully removed from the base of the walking legs using fine forceps. Excised gills were mounted in sea water (S = 35), on a microscope slide without a coverslip, and photographed under low power magnification (× 10 – 40) (Nikon D7000; Niko SM2800 using a camera attachment and cold light source (Schott, KL1500)). Each digital image was calibrated using a stage

micrometer (100 x 0.1 = 10 mm, Graticules Ltd, Tonbridge, Kent, England) and areas were calculated using Image J.

Total gill area was expressed as the sum of the individual areas (×2) and regressed after double logarithmic transformation on body dry mass using the method of least squares.

Dry body mass was estimated by interpolation from a regression equation fitted to log wet mass/log dry mass data (see Supplementary materials 3).

Extrabranchial gas exchange surfaces

The same technique used to measure gill area was also used to estimate surface areas of excised coxal plates of *P. brevicornis* (n = 5). Based on the fact that these plates were so thin, and there is evidence of their being used in gas exchange in other amphipods, they were considered putative extrabranchial gas exchange surfaces (EGS) (see Supplementary materials 4).

Evidence for respiratory pigment in the haemolymph

Haemolymph was obtained by direct cardiac puncture from individuals of all species, except *Probulisca ovata*. Even the largest individual of *P. ovata* was too small to haemolyph sample. Haemolymph was collected using a microsyringe (Hamilton, vol. = 10 or 25 µL), the needle of which was inserted dorsally between the 3 and 4 pereon segments. Samples were transferred to a microcentrifuge tube (Eppendorf vol. = 0.5 mL) on ice. While individual haemolymph samples were obtained for *P. miersi* (15 – 60 µL), because of the small volumes obtained from *Prostebbingia* and *Shraderia* (0.5 – 6 µL per individual) it was necessary to pool samples. Haemolymph samples were then diluted 1:100 and their absorbance over the range λ = 260 – 600 nm determined using the scan function of a UV-VIS spectrophotometer (Helios Gamma, Thermo

spectronic, Cambridge). Both samples and blanks were tested in precision microcuvettes (Quartz SUPRASIL Hellma, 1 cm pathlength).

Rates of crawling and swimming

The activity rates of 10 individuals of the three largest species, *P. miersi*, *P. brevicornis* and *S. gracilis* were recorded at different test temperatures (nominally 0.0, 3.0, 6.0 and 9.0°C) to investigate relationships between the thermal response of both crawling and bursts of rapid swimming with measures of respiratory performance. Initial trials showed that only *P. brevicornis* moved using both burst swimming and crawling. *P. ovata* was too small to reliably track its activity from the video recordings.

Swimming and crawling speed was measured as follows. A 70 mm tall plastic ring (height = 70 mm tall, circumference = 0.82m) was placed into sea water of 40 mm depth. This ring was placed on a 50 x 50 mm grid so that swimming and crawling speed could be calculated from video recordings. Recordings were made using a LifeCam Cinema HD video camera (Microsoft) at 25 frames.sec^{-1} using VideoVelocity 3 software (Candy Labs). This software stamped a time code onto the video recording that allowed swimming speed to be measured by manually plotting the distance moved between frames of known time intervals, where distance was calibrated from the closest grid line to the amphipod. Up to 10 swimming or crawling sequences were captured and analysed for each individual. Individual body lengths were measured and swimming and crawling speeds expressed as units of body lengths per second.

Seawater temperature was maintained within a jacketed aquarium (as described previously) and the temperature raised from ambient (i.e. 0.0 ± 0.2°C, mean ±1SD) at 0.1°C.h$^{-1.}$ Once the test temperature was achieved it was held for 24 h, allowing the

physiology of individuals to stabilise to the test temperature, before swimming trials were conducted.

Results

$\dot{V}O_2$ and ventilation during hypoxia

Patterns of $\dot{V}O_2$ and ventilation during acutely declining PO_2s, for each of the four amphipod species, are presented in Figure 1 and the calculated RI for each species in Figure 2. There was a significant effect of body size on RI ($F_{1,35} = 4.84$, $P = 0.035$) but also a significant effect of species identity ($F_{3,75} = 41.57$, $P < 0.001$) In summary respiratory performance under hypoxia decreased with increasing body size with *P. brevicornis* as an exception.

The species with the largest body size, *P. miersi* showed no oxyregulation and there was possibly evidence of hypometabolism in some of the largest individuals. *P.miersi* therefore had the lowest calculated RI, and while RI appeared to increase with decreasing body size *within* the species when tested this was not significant ($F_{1,11} = 0.25$, $P = 0.626$). It mounted a poor hyperventilatory response (max. 12 % increase at 60 – 55 % a.s.) which remained almost unchanged, even when in anoxia. The smallest species *P. ovata* possessed the strongest pattern of oxyregulation and so the highest calculated RI (Fig. 2). Unfortunately it was not possible to quantify its hyperventilatory response as the pleopods were not fully visible under experimental conditions.

Both *P. brevicornis* and *S. gracilis* were characterised by body sizes smaller than the largest *P. miersi* but larger than *P. ovata* and displayed oxyregulatory patterns intermediate to the smallest and largest species examined. Unlike *P. miersi* both *P. brevicornis* and *S. gracilis* showed a pronounced hypoxia-related hyperventilation.

However, the larger species, *P. brevicornis*, had a significantly greater calculated RI (controlling for body mass) and showed a slightly better developed hyperventilatory response, than the smaller species *S. gracilis*. These patterns of oxyregulatory ability, calculated RI and hyperventilation response were consistent even when individuals of similar body sizes (for all but the smallest species *P. ovata*, where there was no overlap in body mass range) were compared.

There was a significant effect of species on normoxic rates of O_2 uptake. *P. brevicornis* had a significantly higher O_2 uptake than either *P. miersi* or *S. gracilis* (Table 1, Supplementary materials 2.2). The species-specific difference in mass standardised O_2 uptake was driven by *P. brevicornis* alone which had a significantly greater rate than similar sized individuals from the other two (and probably all of the other) three species (Supplementary materials 2.2).

The total gill areas of *P. miersi*, *P. brevicornis* and *S. gracilis* all scaled with body mass and with a similar exponent to the three temperate marine gammarid species investigated by Moore and Taylor [38]: however, the Antarctic species had a consistently smaller mass-specific gill area (Fig. 3). There was no significant difference between the body mass-gill area relationships of the three species (ANCOVA $F_{2,23}$ = 0.65, P = 0.53). If, however, the area of the putative extrabranchial gas exchange surfaces (EGS) on the inside thin surfaces of the coxal plates of *P. brevicornis* is taken into account (Supplementary materials 4) the EGS would increase the total gas exchange surface of *P. brevicornis* by an average of 80 % (range 65-128.3 %) for an individual with a mean dry mass of 22 mg.

Putative respiratory pigment

Presented in Figure 4 are spectrophotometric scans of haemolymph from three of the four species: *P. ovata* proved too small to sample. A peak was detected at λ = 332 nm for hemolymph from *P. brevicornis* but not from *S. gracilis*. The peak disappeared when haemolymph was aspirated with nitrogen gas and partially reappeared when the hemolyph was aspirated with air, suggestive of reversible O_2 binding to the Cu-bearing respiratory pigment hemocyanin (see Supplementary materials 5 for further details). A peak was detected at λ = 433 nm in *P. miersi* hemolymph but was unaltered when hemolymph was aspirated with nitrogen gas. This peak was identical to the carotenoids peak isolated from the brown seaweeds that this species regularly fed on (JIS, unpubl.).

Crawling and swimming behaviour

All three species exhibited quite different crawling responses ($F_{2,107}$ = 9.3, P = 0.003) with different responses to increasing temperature ($F_{2,107}$ = 28.8, P < 0.001) (Fig. 5). Separate ANOVAs for each species followed by *posthoc* Tukey tests for each temperature showed that the crawling speed of *P. brevicornis* was faster at 0.1 and 3.1°C than at 6.5 and 9.6°C (T > 5.0, P < 0.001; Fig. 5A) and faster at 4.4°C than 9.6°C (T = 4.6, P < 0.001). The burst swimming speed of *P. brevicornis* was fastest at 3.1°C (T > 3.6, P < 0.008; Fig. 5B) but speeds also fell precipitously to be slowest at 6.5 and 9.6°C (T > 3.0, P < 0.037). Temperature significantly increased crawling speed of *P. miersi* ($F_{4,53}$ = 3.2, P = 0.033) but crawling speed was not significantly different in pairwise comparisons (T < 2.8, P > 0.060). Crawling speed in *S. gracilis* was considerably slower, at about one third that of *P. brevicornis* and *P. miersi*, and did not

change with temperature ($F_{2,21}$ = 0.88, P = 0.43). All individuals of *S. gracilis* died at T = ~6°C.

Discussion

Support for the Oxygen Limitation Hypothesis

There is some support for the Oxygen Limitation Hypothesis in our study. The largest species *Paraceradocus miersi* did not perform as well (i.e. poor ability to maintain respiratory independence and mount a hypoxia-induced hyperventilatory response) under acutely declining PO_2 as the smallest species *Probolisca ovata.* The performance of the two intermediate-sized species lay between these two extremes. While there was no difference in the allometric relationship between total gill area and body size between the three species investigated, the total gas exchange surface of *Prostebbingia brevicornis* increased markedly if extrabranchial gas exchange surface area was added to total gill area. That said, gill surface areas were consistently smaller (controlling for mass) than the only other aquatic amphipod species for which we have data, i.e. temperate shallow-water gammarids [38].

Qualifications to support for the Oxygen Limitation Hypothesis

The support that our study lends the Oxygen Limitation Hypothesis requires qualification. Firstly *Prostebbingia brevicornis* displayed a markedly better performance under hypoxia than the relatively closely-related, and slightly smaller, *S. gracilis*, when controlling for body size. This better performance could be associated with (1) the best developed hypoxia-related ventilatory response measured, (2) putative extrabranchial gas exchange surfaces which presumably markedly increase the total area available for gas exchange, and (3) the presence of the respiratory

pigment hemocyanin, which carries and reversibly binds O_2. Such innovations presumably enhance the ability of *P. brevicornis* to obtain and transport O_2 to the tissues. Such a claim is strengthened by the fact that *P. brevicornis* has a markedly higher $\dot{V}O_2$, and is capable of considerably faster movement and swimming than similarly-sized *S. gracilis* and *P. miersi*. So with respect to the prediction of the Oxygen Limitation Hypothesis that *all else being equal* reduced O_2 should have disproportionately larger effects on the performance of large-bodied individuals, this current work suggests that in the case of *P. brevicornis* all else is not equal. This species has respiratory adaptations likely linked with a comparatively good respiratory performance under declining PO_2s and, compared with the other species studied, a very active lifestyle. Thus, it possesses a better respiratory performance than the Oxygen Limitation Hypothesis would predict.

Secondly, there were only small improvements in respiratory performance in small individuals compared with larger individuals of the two largest species, *P. miersi* and *P. brevicornis*. These differences were not, however, statistically significant. These findings arguably lessen the support for the generality of the Oxygen Limitation Hypothesis.

Support for the Symmorphosis Hypothesis

As discussed, the respiratory performance and associated adaptations with respect to gas exchange and transport of *P. brevicornis* provides an exception to the Oxygen Limitation Hypothesis. However, they do lend some support to the Symmorphosis Hypothesis [9]. The ‘exception’ of *P. brevicornis* fits with the prediction that effects of reduced O_2 should be independent of body size: Compared with *S. gracilis* the respiratory system of *P. brevicornis*, seems the subject of evolutionary change

(development of extrabranchial exchange surfaces, and respiratory pigment) that could compensate for body size limitations. If true, *P. brevicornis* has been shaped to supply adequate O_2 for metabolism in a species, which, unlike the other three species, displays a very active lifestyle in what is generally considered a very sluggish environment. The principles of symmorphosis are also supported by the marked decline in swimming performance of *P. bervicornis* at temperatures above 3°C, which supports the notion that its respiratory system has evolved to maintain swimming speed in the constant cold of the Southern Ocean temperatures and cannot supply the demands of its active lifestyle at warmer temperatures. The fact that there was such a small (and non-significant) effect of mass on the respiratory performance of *P. brevicornis* and *P. miersi* under acutely declining O_2, even over such a wide range of body sizes tested, could also be seen as supporting the notion of symmorphosis. Testing symmorphosis is notoriously difficult, but some of our findings are at least consistent with the hypothesis.

Support for the Respiratory Advantage Hypothesis

Our study also offers some support to the 'Respiratory Advantage Hypothesis' [10]. The largest, and arguably only 'giant', species we examined, *Paraceradocus miersi*, had little or no oxyregulatory capacity, poor ventilatory ability showing little response to hypoxia, and did not appear to have an O_2-binding pigment like hemocyanin or haemoglobin dissolved in its hemolymph. This is consistent with the idea that large body size in an animal which does not have well developed mechanisms for respiratory regulation bestows a respiratory advantage that can overcome viscosity-related, low O_2 availability in polar waters and the prediction that gigantism should be absent in polar groups characterised by good respiratory control.

However the second prediction, that reduced O_2 should have a disproportionately large effect on the performance of small-bodied individuals does not seem to hold gains little support from our study. Smaller individuals of *P. miersi*, equivalent in size to *Prostebbingia brevicornis* and *S. gracilis* did not have a significantly different oxyregulatory ability from (and had a similar ventilatory response to declining O_2) to the largest individuals of the same species. Similarly large individuals of *P. brevicornis* displayed the same respiratory performance under hypoxia as did smaller individuals.

Conclusions and perspective

This study provides qualified support for the Oxygen Limitation Hypothesis of Chapelle and Peck [8], but also some degree of support for the Symmorphosis Hypothesis [9] and perhaps even the counter-intuitive Respiratory Advantage Hypothesis [10].

We suggest on the basis of the support we found for the Oxygen Limitation Hypothesis that giant polar amphipods are likely be some of the first to fare badly in an O_2-poor ocean. However, the products of past evolutionary innovation, such as respiratory pigments that enhance O_2- transport and novel gas exchange structures, in some if not all species, may, to some extent, offset any respiratory disadvantages of either large or small body size, consistent with the Symmorphosis hypothesis. We suggest that the effects of O_2 reductions on polar amphipods, and possibly other marine groups, depends on the composition of individual biologies and not just body size [21,25]. For example knowledge of mechanisms comprising the respiratory biology of species, and perhaps even individuals (e.g. ventilation, perfusion, oxygen transport, cellular metabolism, anaerobic capacity) and how those mechanisms respond to, and can be induced by, environmental drivers is invaluable in understanding relationships between O_2 and body size. This is particularly so for species which do not appear to

have structures we naturally associate with particular functions, and yet the functions are present nevertheless, e.g. hypoxia-sensitive gut movements in Antarctic pycnogonids [32] and free-living brine shrimp embryos [40] which generate additional circulatory function helping to produce (or maintain) internal O_2 diffusion gradients. So too is knowledge of factors affecting oxygen supply (e.g. water current, degree of hypoxia encountered *in situ*) and demand (scope for activity, behaviour of movement, feeding and growth rates). The key is to identify which factors are most important in terms of their influence on multi-species comparisons, and either incorporate or control for them.

In this present comparison we are still left with the puzzle of why *P. brevicornis* should have evolved greater burst and crawling speed than the other species investigated, and how these innovations might be linked to respiratory innovations that appear to be absent from the other three. The explanation clearly lies in the natural history of the species, e.g. predation pressure, food acquisition. Currently this natural history, as with much Antarctic life, is largely unknown.

It is tempting to assume that a greater number of closely- related species (or at least species with a well resolved phylogeny), encompassing a wide range of body sizes, *on its own* would have produced a stronger test of the predictions of the three hypotheses posited. However, this study has highlighted it is not just number but choice of species that is important. Treating species as statistically independent replicates, ignoring their phylogeny, has been shown (at least sometimes in physiological studies) to have its difficulties (e.g. [39]). However, even more so ignoring the product of that evolutionary divergence, the resultant adaptations and convergence, as integrated in individual species is problematic. When testing macrophysiological hypotheses it may even lead to spurious patterns and imperfect

tests of unifying hypotheses. Taking such products into account should instead strengthen such multi-species comparisons and meta-analyses.

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Data Accessibility

The datasets supporting this article will be uploaded as part of the Supplementary Material.

Authors' Contributions

Both authors contributed to the conception and design of the experiments, and the acquisition, analysis and interpretation of the data. Both authors contributed to drafting, revising and approving the submitted manuscript.

Competing Interests

We have no competing interests.'

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Figure Legends

Figure 1. Rates of O_2 uptake and ventilatory activity of four Antarctic species exposed to acutely declining environmental PO_2. Values and means ±1 S.D. (n = 6 in each case). Dashed line = line of oxyconformity.

Figure 2. Regulation Index for each of the four Antarctic amphipod species studied, indicating the phylogenetic relatedness of the species, and the body size categories (wet mass) of all individuals examined. All data are normally distributed (Anderson-Darling ≤ 0.297, P ≥ 0.347) and variances were not significantly different (Multiple Comparisons, P = 0.347). M = *P. miersi*, B = *P. brevicornis*, G = *S. gracilis*, O = *P. ovata*.

Figure 3. Relationship between total gill area and dry body mass of three of the Antarctic amphipod species together with Moore and Taylor's [38] data on the gill areas of some temperate marine gammaridean amphipods. Dashed line = line of best fit $\log_{10}y = 0.813 \log_{10}x + 0.294$, $r^2 = 90.52\%$). Each of the solid lines are lines of best fit for (from top to bottom) *Echinogammarus pirloti*, *Gammarus locusta* and *Gammarus duebeni* from Moore and Taylor [38].

Figure 4. Spectrophotometric scan of untreated haemolymph from three Antarctic amphipod species A. *P. miersi*, B. *P. brevicornis* and C. *S. gracilis*. Solid line = air saturated. Broken line = deoxygenated.

Figure 5. Crawling (A) and burst swimming speed (B) of three Antarctic amphipod species. Values are means ±1 S.D. Both crawling and swimming speeds were Log_{10} transformed to normalise residuals (Anderson-Darling < 0.403, P > 0.333). Crawling speeds had equal variances (Levene's test, P > 0.058).

Figure 1

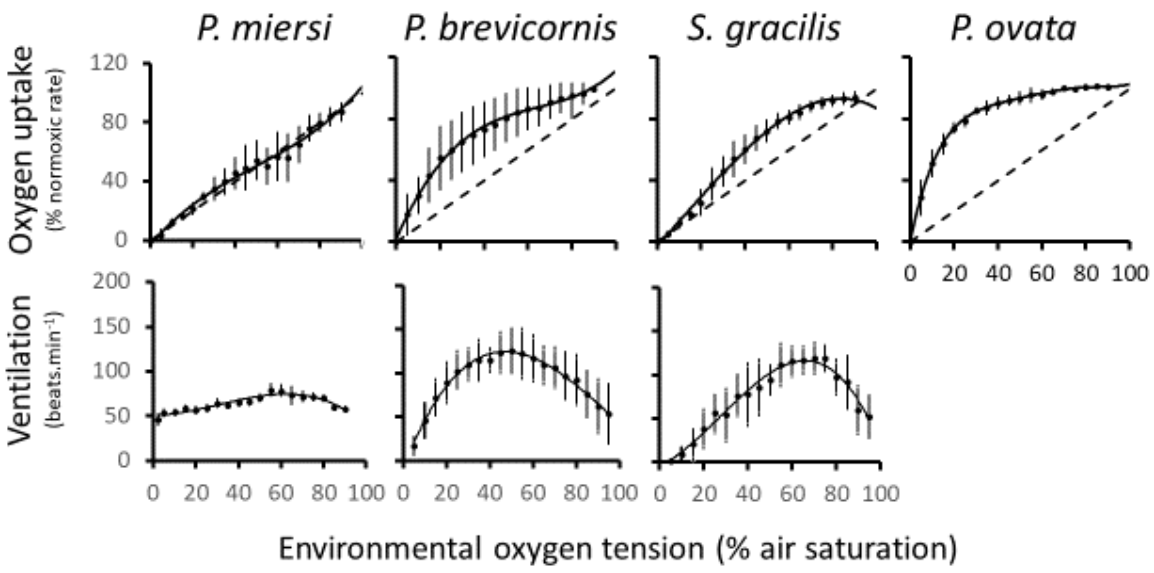
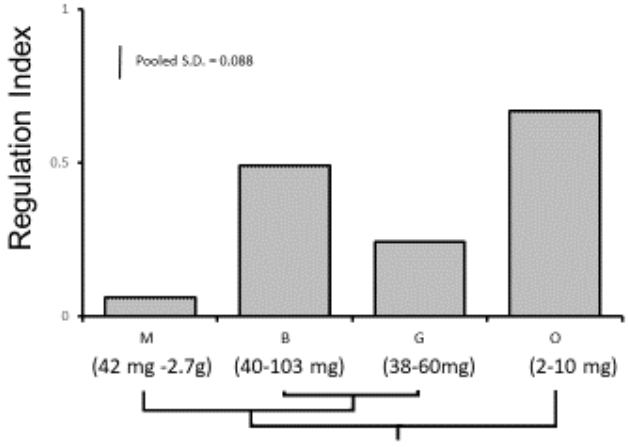


Figure 2



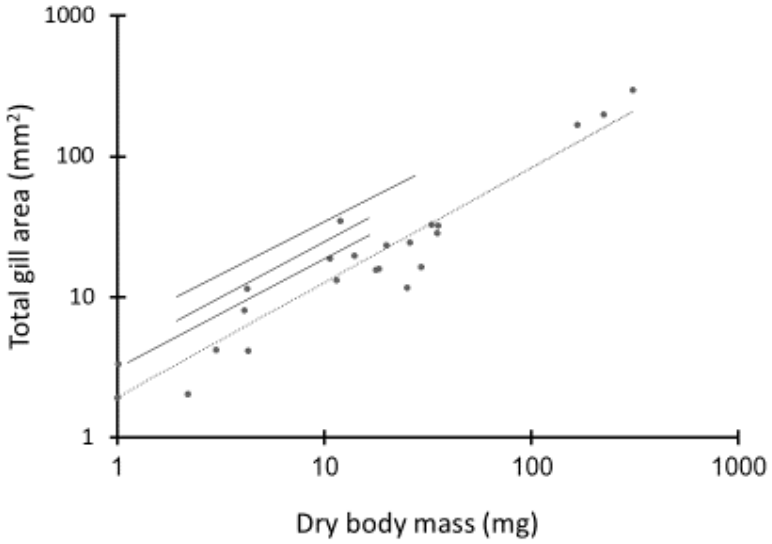


Figure 3

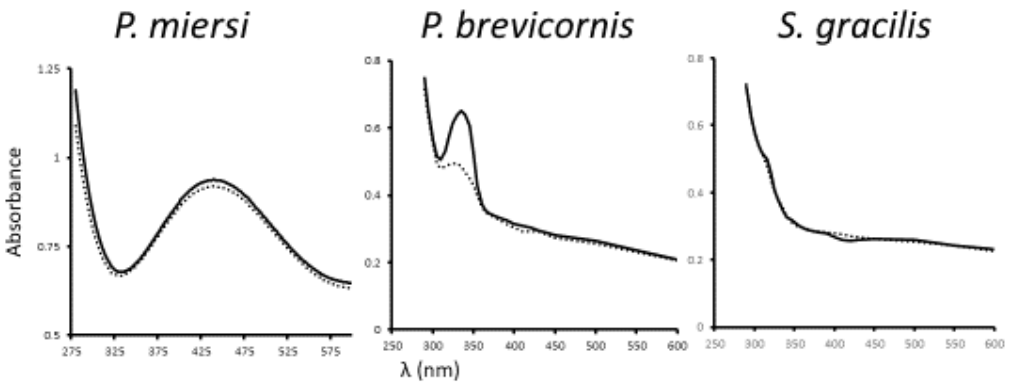


Figure 4

Figure 5

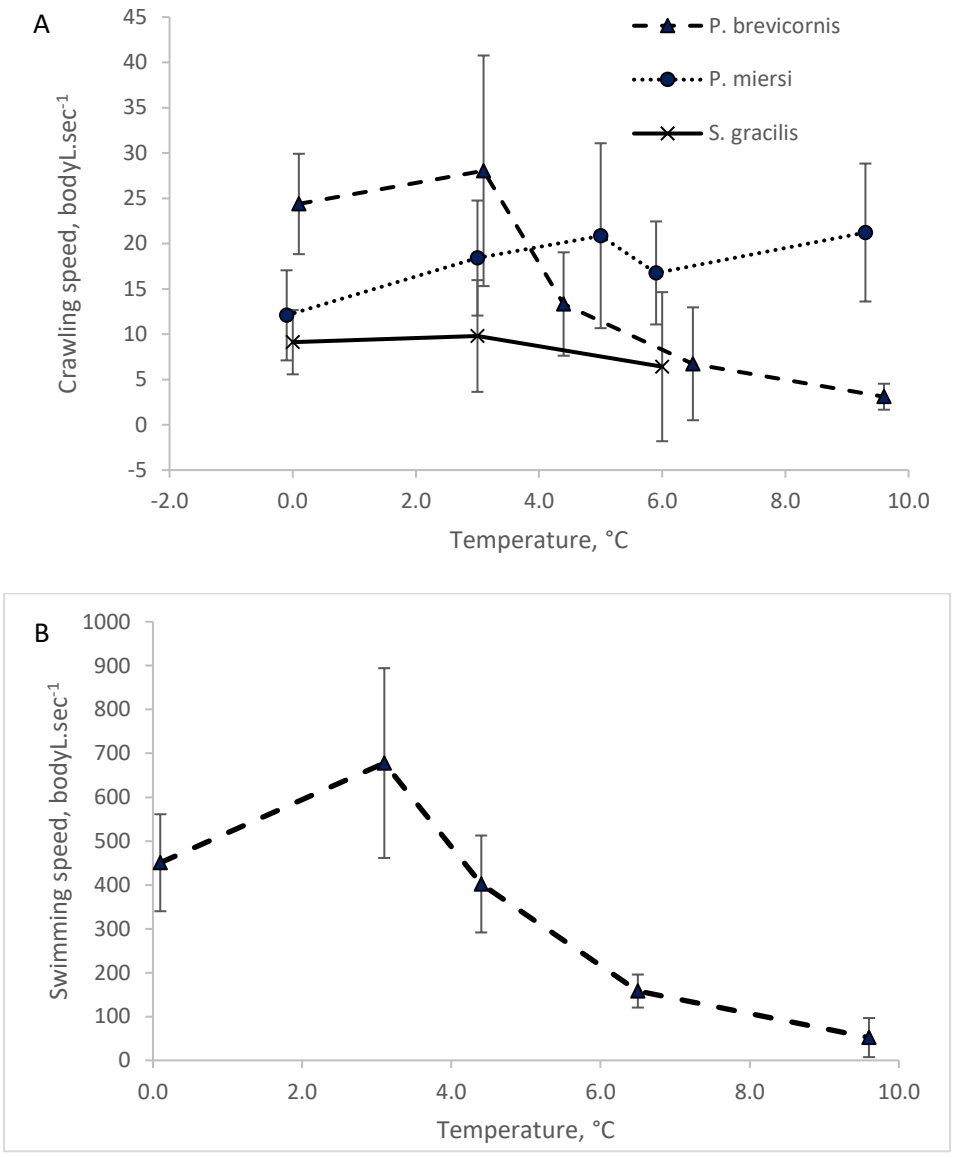

A
P. brevicornis
P. miersi
S. gracilis
Crawling speed, bodyL.sec-1
Temperature, °C
B
Swimming speed, bodyL.sec-1
Temperature, °C

Table 1. Mass-specific rates of O_2 uptake ($\dot{V}O_2$) for different mass ranges of four amphipod species investigated. Only those marked with an asterisk have similar body masses, and so have comparable O_2 uptake values (Welch's test $F_2 = 0.49$, $P = 0.629$) although see Supplementary materials 2. *P. brevicornis* had a significantly greater $\dot{V}O_2$ than small *P. miersi* and *S. gracilis* (ANOVA $F_{2,17} = 4.75$, $P = 0.026$).

	Wet mass mean ± 1 S.D. (range)	$\dot{V}O_2$ (uL $O_2.h^{-1}.g$ wet $mass^{-1}$)
Paraceradocus miersi	1.79 ±0.76 g (1.14 – 2.77)	26.24 ± 3.78
	51.98 ± 15.64 mg* (36.7 – 74.0)	58.12 ± 2.47
Prostebbingia brevicornis	55.89 ± 11.68 mg* (33.7 – 67.0)	68.05 ± 10.31
	118.76± 10.96 mg (108.0 – 133.0)	54.28 ± 4.16
Shraderia gracilis	49.3 ± 10.59mg* (36.5 – 62.8)	55.18 ± 5.50
Probulisca ovata	2.67 ± 0.53 mg (2.3 – 3.4)	103.3 ± 14.95

Supplementary materials 1

Amphipod collection, species and habitat (with particular reference to oxygen) details

Collection Site

Collection of animal material was carried out on four separate days during the austral summer; 21st January and the 6th, 8th and 11th February, 2007. Amphipods used in the experiments reported here were collected by divers from beneath cobbles/boulders at a depth of 6 - 8 m from South Cove, Ryder Bay, Adelaide Island, Graham Land, Western Antarctic Peninsula (lat. 67°34'11''S, long. 68°08'89'' W) (Fig. S1.1). The sea floor here is primarily natural bedrock and rubble (granite), with variable patches of fine biogenic and mineral debris (Fig. S1.2 below). The site has a score of 0 on the Wentworth [1] Classification Scale that quantifies rugosity of substrate.

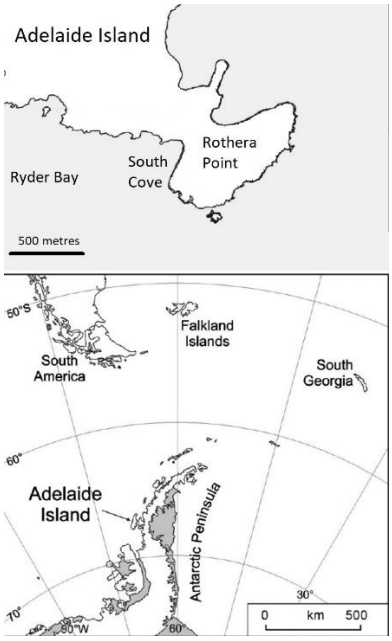


Figure 1.1 Location of the collection site (South Cove) and Rothera point on Adelaide Island (top panel) and Adelaide Island relative to the Western Antarctic Peninsula (bottom panel)

It is a south-facing site which means that the main disturbance encountered is ice scouring and impact. The collection sites, which comprise IBIS sampling grids come under the Rothera Oceanographic and Biological Monitoring (RaTS) programme.

The sites are typically covered by winter fast ice for *circa* 3 months each year. Water temperatures range from -1.9 to +1.5 m at 15 m depth (RaTs), with the daily and weekly variability in December five times that in July.

Collection method

Amphipod collection was by means of the Souster and Yates Suction Sampler [2]. The bespoke device is driven by a submersible bilge pump (Xylem ISO 8849) which filters 31.5 $L.min^{-1}$ of sea water through a Professional hand held net Bag (EFE & GB nets, Totnes, 1 mm mesh size) in which the amphipods are retained. Divers would turn over larger boulders and vacuum the animals beneath them with the sampler. The Sampler was returned to the laboratory within 20 min of sampling, the net bag removed, and the amphipods transferred (using plastic tea strainers) to the aquarium facilities in the Bonner Laboratory, Rothera field station.

Animal material collected

The four species presented in Fig S1.2 are those chosen for our study and were extremely common beneath the cobbles and the stones in South Cove (Fig. S1.3A), and are also common in similar habitats on the Antarctic Peninsula and beyond.

Figure S1.2 Species investigated in this study. (From top to bottom), *Paraceradocus miersi, Prostebbingia brevicornis, Schraderia gracilis, Probolisca ovata*

Paraceradocus (Megamoera) miersi (Pfeffer, 1888)

(Family Maeridae [n.b. De Broyer et al.[3] places it in the family Melitidae]; Superfamily Hadzioidea; Suborder Senticaudata)

The individuals we used match very closely the pictures of this species presented in Souster [2], the identity of which was confirmed for Dr Souster by Dr. Anna Jazdzewska (p. 48)

P. miersi is a detritivore/scavenger which lives under stones in burrows in sediment which they excavate ([4]; also see Fig. S1.3C below). It is a large species and occurs in east and west Antarctic Provinces and South Georgia district (0 - 344 m depth) [3].

Prostebbingia (*Pontogeneilla*) *brevicornis* (Chevreux 1906)

(Family Pontogeneiidae; Superfamily Calliopioidea; Suborder Senticaudata)

P. brevicornis occurs in west Antarctic province, South Georgia district and the sub Antarctic Islands province (0 -310 m depth) [3]. The individuals we used match very closely the pictures of this species presented in Souster [2], the identity of which was confirmed for Dr Souster by Dr. Anna Jazdzewska (p. 48). Interestingly she records the 'species' as '*P. brevi/longicornis'*. In the manuscript we have used *P. brevicornis*, though it should be kept in mind that an expert in the systematics of the group opted for this dual 'identity'. *P. brevicornis*, together with *S. gracilis* (below) have been recorded previously from coarse sediments where macroalgae present, and are considered herbivores and detritivores [5].

Schraderia gracilis Pferrer, 1888

(Family Pontogeneiidae; Superfamily Calliopioidea; Suborder Senticaudata)

S. gracilis occurs in east west Antarctic province, South Georgia district and the sub Antarctic Islands province (0 - 338 m depth) [3]. The individuals we used match very closely the pictures of this species presented in Souster [2], the identity of which was confirmed for Dr Souster by Dr. Anna Jazdzewska (p. 48).

Probolisca (*Metopa*) *ovata* (Stebbing, 1888)

(Family Stenithoidae; Superfamily Amphilochoidea; Suborder Amphilochidea)

The smallest species investigated is distantly-related to the other amphipods and occurs in west Antarctic province, South Georgia district and the sub Antarctic Islands province (0-570 m depth)) [3].

Environmental oxygen (O_2) status at the collection site

Unfortunately the RaTS monitoring scheme does not include dissolved O_2 as one of the parameters it measures (see the official website for what it does cover at https://www.bas.ac.uk/project/rats/#about).

The O_2 saturation of water overlying the boulder field and of water drawn from within presumed *Paraceradocus* burrows, excavated beneath boulders slightly embedded in the sea bottom (Fig. S1.3), was measured using a Presens system on water samples collected on the 6th and 8th February 2017 as follows.

To obtain burrow water the following procedure was carried out. A team of divers carrying 4 x 20 mL thick plastic syringes each fitted with a 30 cm length of gas impermeable tubing (Tygon©) entered the water and descended to the boulder field. Firstly divers would locate possible burrows beneath large boulders either by making visual contact with *Paraceradocus* at the entrance to the burrow, or by coming to

recognise the distinctive depression in the sediment below particular boulders (Fig. 1.3B). The tube attached to the syringe would then be carefully inserted into the potential burrow, to a depth of > 10 cm avoiding disturbance of any sediment. Slowly the syringe was filled with burrow water. Immediately the syringe was full of water, the tube was quickly extracted from the burrow and the end was sealed with a plastic clip to avoid leakage from the burrow water sample. The boulder was then overturned so that any amphipod species in the burrow or in other spaces beneath the rock could be checked visually and their presence and behaviours noted (Fig. 1.3C). Sealed water samples were returned to the surface, and analysed on the boat < 7 min after collection.

Water overlying the boulder field was sampled from the dive boat, from a depth of 30 cm below the surface, and the O_2 content determined < 30 sec after collection.

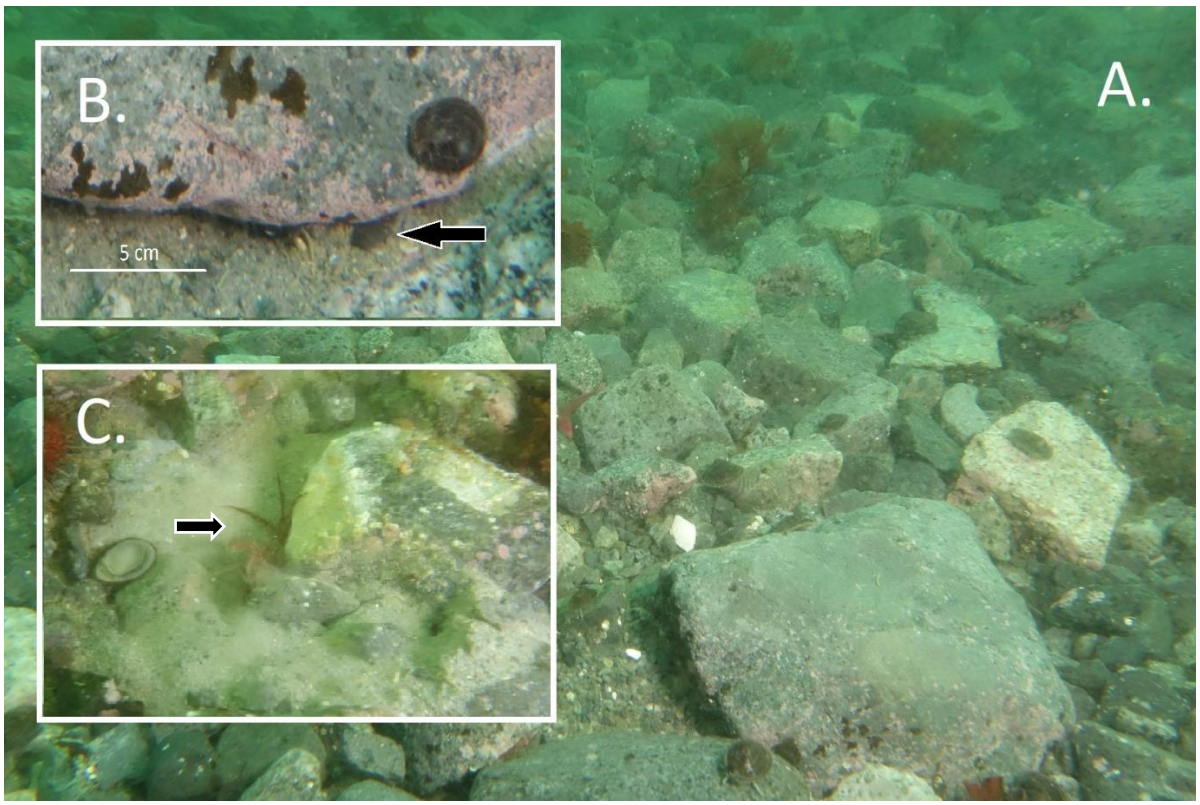


Figure S1.3 The sampling site comprised cobbles and boulders in relatively shallow water around 4-6 m depth (A). Paraceradocus *burrows could be located either by the presence of the animal at the mouth of the burrow or by the depression in the biogenic and mineral sediment beneath, and to the side of, the boulders (B – arrow marks opening to burrow). When the boulder was removed, it was often possible to see the* Paraceradocus *in its typically inverted position, still lying within its burrow (C – arrow marks animal in its burrow).*

Presented in Figure S1.4 is the O_2 saturation (% a.s. or air saturation) of sea water collected from different conditions in South Cove. During the sampling period the overlying sea water was hypersaturated with O_2. The O_2 concentration of burrow water from confirmed *Paraceradocus* burrows was substantially lower than overlying sea water but was still around 100 % a.s. saturation. The O_2 content of water filled spaces beneath boulders lying on substrate with a visibly high biogenic content were substantially lower than that in the burrow water.

In summary while the overlying water was oversaturated with O_2, O_2 conditions beneath the boulders where the amphipods were collected ranged from mildly hypoxic (beneath boulders) to normoxic (in burrows).

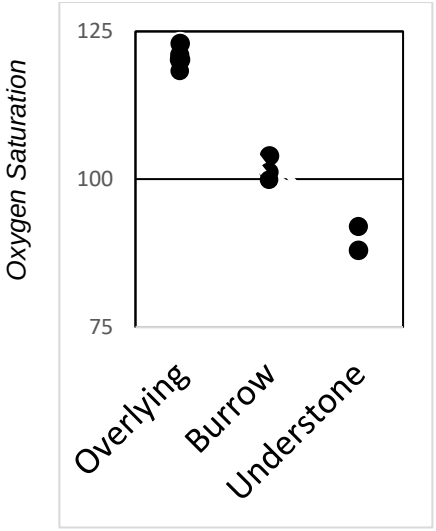


Figure S1.4 O_2 saturation (as % air saturation) of sea water from different conditions in South Cove (8/2/17). Each point is one measurement.

Conclusion: While *Paraceradocus* inhabiting their burrows experienced normoxic conditions smaller individuals or other smaller species living under boulders may have experienced exposure to mildly hypoxic conditions.

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Supplementary materials 2

Respirometry

A. Comparing closed and semi-open respirometer techniques

For the closed respirometry which was carried out at Rothera, Antarctic Peninsula and also as part of the comparison of techniques at the University of Plymouth glass bottles (volumes = 100, 60, 25, 11, 5 and 1.8 mL), each fitted with a gas-tight lid and an oxydot were used as respirometers. The size of the bottle was matched to the size of the individual such that the time course of the rate of O_2 decline with time in each bottle was broadly comparable. Each bottle contained an appropriately scaled section of plastic mesh inserted (5 cm x 5 cm, 4 cm x 4 cm, 3 cm x 3cm, 4cm x 2 cm or 2 cm x 1 cm respectively, mesh size = 1 mm in each case) to which the amphipod could adhere throughout the experiment. Providing a substrate to which amphipods can adhere or shelter within substantially reduces amphipod activity as shown previously for temperate [1] and Antarctic [2] species. Each bottle was also fitted with a small Teflon covered metal stirring bar, separated from the amphipod by plastic mesh. The actual volume of water within each bottle was calculated by subtracted the volume of water displaced by the animal, the stirring bar, and the plastic mesh.

Bottles without lids were submerged in jacketed water baths (22 x 40 x 75 cm) each containing constantly aerated sea water maintained at the appropriate test temperature (T = -1.0 ± 0.1°C) using thermostatically controlled water baths that pumped cooled water through the jacket (Grant LT D20G). One amphipod was carefully placed in each bottle the night before measurements commenced. Gauze was secured across the mouth using a cable tie. This prevented the individual from escaping while allowing free exchange of dissolved gases with water in the bottle. The next morning the gauze was removed and replaced with a gas-tight lid, fitted with an extra layer of aluminium foil. The whole operation was carried out with the bottles and lids submerged, taking care not to introduce air bubbles. Eleven amphipods were run at any one time. One empty bottle was included to quantify background respiration and taken into account when calculating the rate of O_2 uptake for individuals.

This closed technique for measuring oxygen uptake of Antarctic amphipods was compared with a semi-open technique used previously for crustaceans, including amphipods. The reason for this was that open and semi-open techniques are often seen as superior to closed bottle techniques for a number of reasons (e.g. the animal is more settled, and the water is not fouled so quickly using a semi-flow through technique) but the closed bottle technique is easier to use and so was preferred for use in Antarctica.

The semi-open respirometry technique run alongside the closed bottle technique at the University of Plymouth in September 2016 was set up as follows. Single

amphipods were placed into the same specially constructed Perspex respirometer (vol. = 21 mL) as used by Agnew and Taylor [1] when investigating the effect of declining oxygen tensions on the oxygen uptake of the intertidal amphipods, *Echinogammarus pirloti* and *E. obtusata*. Each respirometer had an inlet and outlet pipe which was connected *via* gas-impervious tubing (Tygon) to a pump situated in a reservoir (vol. = 2L) of filtered, continuously aerated, sea water maintained at T = 0°C (S = 35). Reservoir water was gently pumped through the respirometer supplying the amphipod with clean flowing sea water. Each respirometer was fitted with an enclosed magnetic stirring bar. The amphipod was protected from the bar by a piece of plastic gauze to which it often adhered during its time in the respirometer. Instead of an O_2 electrode (ES046 Radiometer, Denmark) being inserted into the chamber to measure oxygen depletion the same oxydot system as was used in the closed bottle respirometers was used. Amphipods were left overnight (for the same period as those amphipods in the closed system experienced) before the experiment was started. At the end of this period the pump was stopped and the amphipod was left to deplete the O_2, in the chamber as a result of its own respiration. Controls consisting of respirometers without animals were run simultaneously. The rate of O_2, reduction inside the respirometer was recorded and the rates of O_2 uptake and Respiratory Index (RI) calculated exactly as outlined for the individuals in the closed bottle set up. The pH of the sea water in both the closed bottle and the semi-open respirometer decreased < 0.3 pH units during the course of the experiment. However, in line with the checks carried out by Agnew and Taylor [1] during exposure of amphipods to declining O_2, we frequently flushed the chamber with water at the same O_2 tension (prepared using a set of Wostoff precision gas mixing pumps, Bochum, Germany) as recorded in the chamber just before the flushing. The respirometer was then sealed again and the O_2 decline produced by the respiration by the amphipod, continued. This made no difference to the measurements obtained.

The VO_2s of *Prostebbingia brevicornis* and *Paraceradocus miersi* were measured under conditions of declining PO_2s concurrently using closed bottle and semi-open respirometry. The amphipods were collected at Rothera on the Western Antarctic Peninsula (See Supplementary materials 1) during the austral summer (2005-2006) and returned to the UK by ship in specially constructed thermostatically controlled shipping aquaria. They were kept at T =-1 to 0°C in aquaria at the British Antarctic Survey buildings in Cambridge for a couple of months before they were transported by road, in thermostatically controlled containers, to the University of Plymouth. Here they were kept (T = 0°C, S = 35), unfed for at least 96 h, before used in the technique comparison trials.

Presented in Figure S2.1 is the VO_2 response to acutely declining O_2 tensions for one individual of *P. brevicornis* and one individual of *P. miersi*, together with an example of perfect regulation (RI = 1) and an example of perfect oxyconforming (RI = 0).

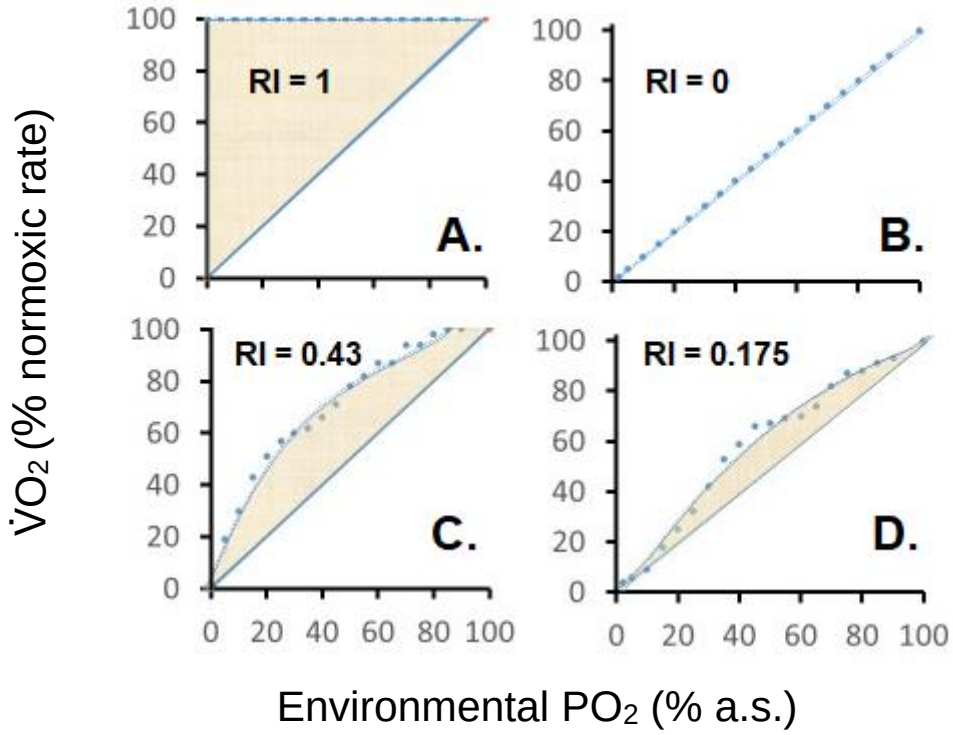

Figure S2.1 Changes in $\dot{V}O_2$ (blue circles) with acutely declining environmental PO_2 for (A) Hypothetical animal with perfect oxyregulation (B) Hypothetical animal which is an oxyconformer (C) Prostebbingia brevicornis and (D) Paraceradocus miersi. The Respiratory Index (RI) is represented by shaded areas, delinated by the oxyconforming line (blue line) and the $\dot{V}O_2$ (blue circles with fitted blue line).

Presented in Figure S2.2 are mean RI and $\dot{V}O_2$ values for both species measured using closed and semi-open respirometry.

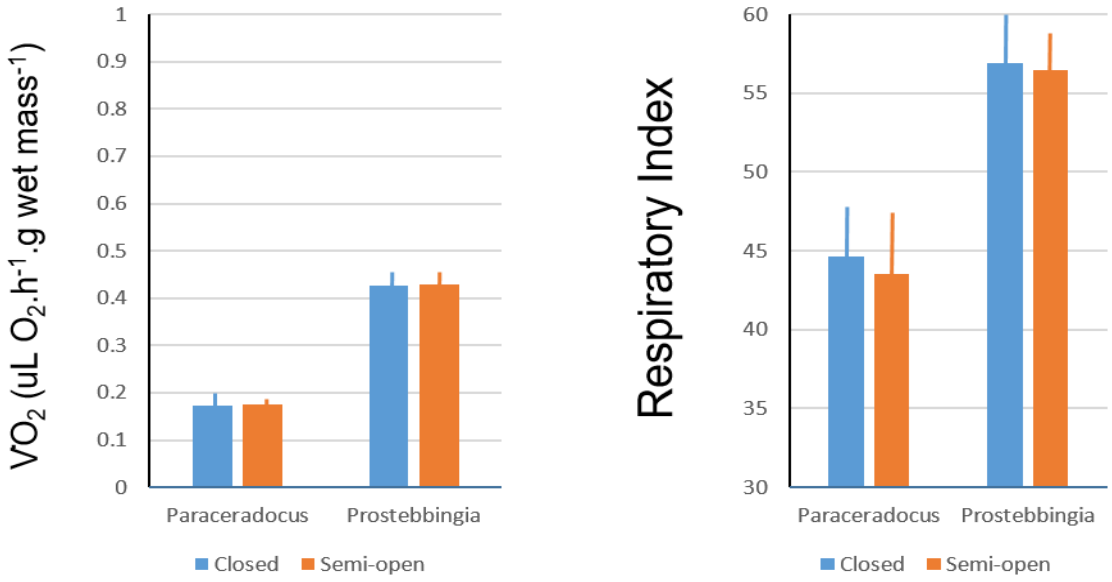

Figure S2.2 in $\dot{V}O_2$ and Respiratory Index for *Paraceradocus miersi. and Prostebbingia brevicornis measured using different respirometry techniques. Values are means ±1. S.D.*

There was no significant difference in the calculated RI for individuals run in the closed bottle system compared with those run in the semi-open system ($t \leq 0.67$, $P \geq 0.522$, $df = 7$). The normoxic VO_2 of *P. brevicornis* was significantly greater than that of *P.miersi.* However, there was no difference as a result of the two test conditions, semi-open or closed respirometry ($t \leq 0.65$, $P \geq 0.534$, $df = 7$).

B. $\dot{V}O_2$ and body mass

The experiments reported in this paper carried out in Rothera, Western Antarctic Peninsula set out to control for, but not investigate, the effect of body mass on mass-specific rates of O_2 uptake ($\dot{V}O_2$). In the main paper we showed that when comparing species of similar body masses, *P. brevicornis* had a significantly greater $\dot{V}O_2$ than either *S. gracilis* or *P. miersi*. However, because the size ranges do not overlap we could not include *P. ovata* in the formal comparison. However, because there were inter and intra-specific mass differences in the individuals used, it is possible to build a supporting circumstantial case for *P. brevicornis* being the only one of the four species to have an elevated $\dot{V}O_2$.

Presented in Figure S2.2 is $\dot{V}O_2$ expressed as a function of body mass for individuals of all four species investigated.

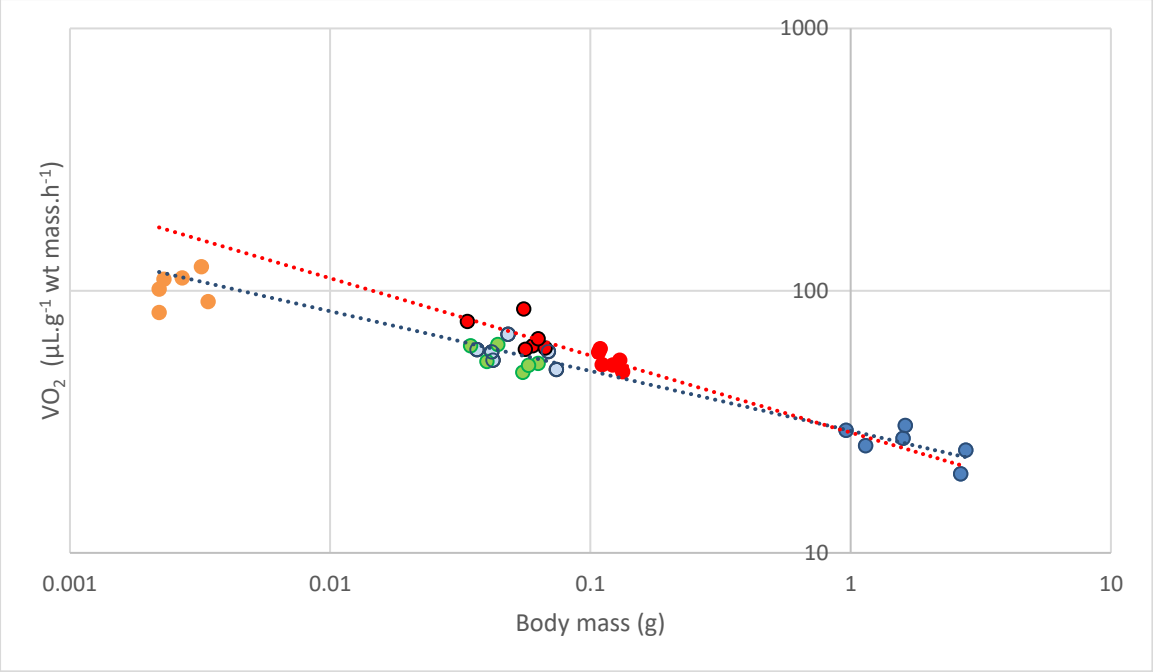

Figure S2.2. $\dot{V}O_2$s for P.miersi *large (dark blue) and small (light blue, with black border),* P. brevicornis *large (red) and small (red with black border),* S. gracilis *(green) and* P. ovata *(orange). Each point represents a single individual. Dashed blue line = line of best fit for all values of* P. miersi *(log_{10} y = 1.466 – log_{10} 0.228x; r^2 = 94.6 %, $F_{1,11}$ = 175.45, P < 0.001) Dashed red line = line of best fit for all values for* P. brevicornis *(log_{10} y = 1.460 – 0.294 log_{10} x; r^2 = 66.3%, $F_{1,11}$ = 19.71, P < 0.001). There were no significant relationships for* S. gracilis *or* P. ovata *on their own ($F_{1,5}$ ≥ 3.76, P ≥ 0.125 in each case).*

There were significant relationships detected for *P. miersi* and *P. brevicornis* but not the other two. The line of best fit for *P.miersi* when extrapolated bisected the cloud of *P. ovata* points. Furthermore correlation which included *P. miersi*, *S. gracilis* and *P. ovata*, improved the r^2 calculated for *P. miersi* on its own from 94.6 to 95.2 %. The relationship between mass and all species with the exception of *P. brevicornis* could be described by the equation $\log_{10} y = 1.468 - 0.212 \log_{10} x$ ($F_{1,23} = 437.39$, $P < 0.001$). There was a significant difference between *P. brevicornis* and all of the other species taken together (ANCOVA $F_{1,35} = 23.19$, $P < 0.001$).

In conclusion, the relationship between $\dot{V}O_2$ and body mass for *P. ovata* fits well with all of the other species except *P. brevicornis*. This together with the formal ANCOVA comparison between *P. brevicornis* and the remaining three species provides strong circumstantial evidence for the statement that *P. brevicornis* has a greater $\dot{M}O_2$ than the other three species investigated, not just the two where the overlap in mass range allowed a straight forward formal comparison.

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Supplementary materials 3

Estimating dry body mass

The wet mass of 10 carefully blotted dry individuals of each species spanning a wide mass range, was measured using a precision semi-microbalance (Genius ME235S, Sartorius, Bradford, MA). They were then oven dried at T = 60 °C to constant mass and reweighed using the same precision microbalance. There was a significant relationship between wet and dry mass detected for each of the three species ($r^2 \geq 0.970$, $P < 0.001$ in each case), with *S. gracilis* having a significantly different relationship from the other two species ($F_{1,29} = 3.57$; $P = 0.032$) The resultant equation for *S. gracilis* is $y = 0.673x + 0.002$, and for *P. brevicornis* and *P. miersii* is $y = 0.2964x + 0.003$ where y = dry body mass (mg) and x = wet body mass (mg).

Supplementary materials 4.

Coxal plates as putative extrabranchial exchange surfaces on *P. brevicornis?*

Coxal plates of P. brevicornis

Figure S4.1 shows the position and shapes of the coxal plates (CP) of *P. brevicornis*. They are noticeably more well developed than *P. miersi* or *S. gracilis* (cf. Fig.S1.2). They are not as well developed as *P. ovata*, but in the case of the latter, which is a tiny species the coxal plates are completely opaque and it is difficult see through or in the plate itself, even when shining light through.

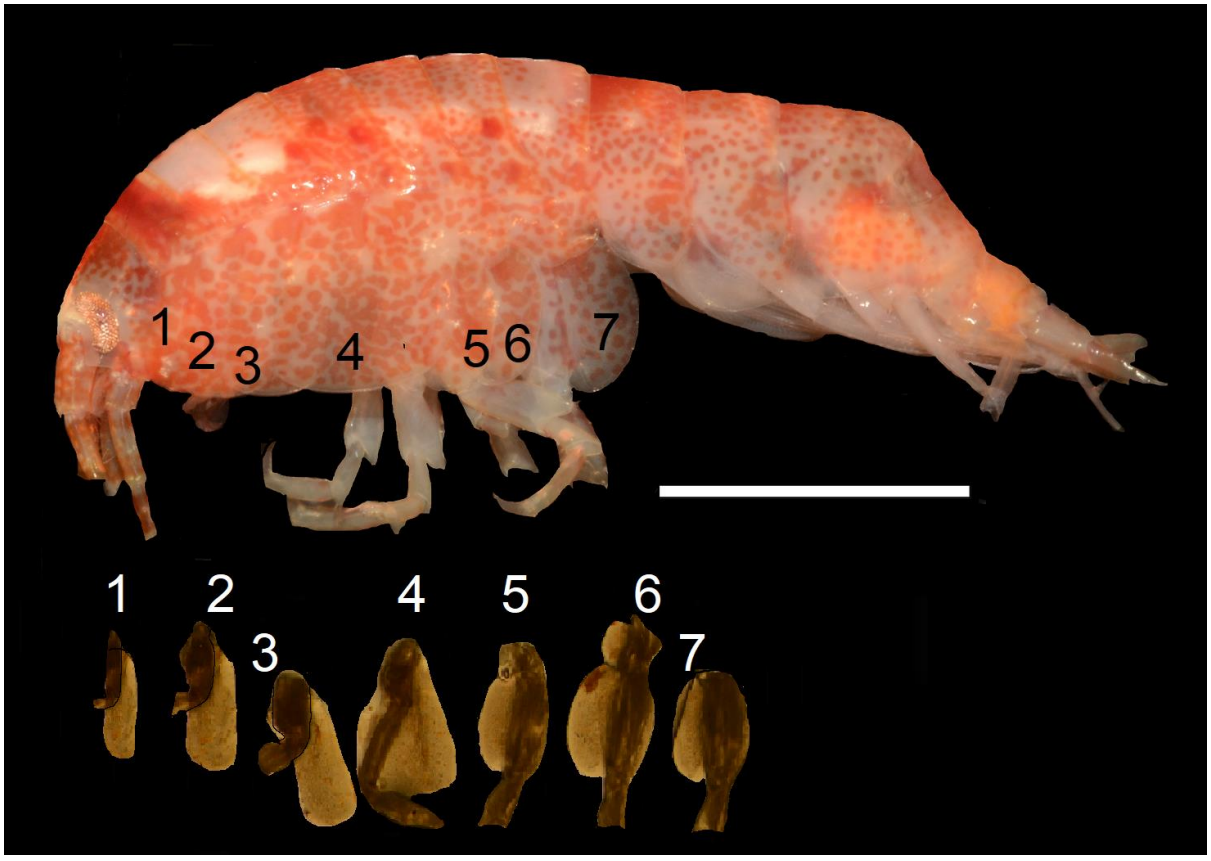

Figure S4.1 Prostebbingia brevicornis with coxal plates 1-7 marked. White line = 5 mm length. Below the main picture are the median (inside facing) surfaces of the excised coxal plate with the first couple of segments of the pereopods still attached. The numbers in white correspond directly with the numbers on the coxal plate marked on the photograph above

Each coxae is broadly a rectangular (CP1-4) or rounded plate (CP5-7), and like all other amphipods, is the highly modified first article of each of the thoracic limbs [1]. Unlike many amphipod species CP 1-4 are, not much larger than CP5-7. For CP1-4 the anterior margin of each plate overlaps one in front of the other. In most amphipods,

but particularly pronounced in the case of *P. brevicornis*, the coxal plates dramatically increase the compression of the body, forming a deep ventral groove, an extensive inner channel for respiratory and other water movements. As in other species this groove houses and protects the gills and (in females) oostegites, both of which are extensions of the thoracic limbs [1].

It is a straight-forward process to remove the coxal plates and measure their surface area using exactly the same procedure as used for the gills (Fig S4.1). For *P. brevicornis* the coxal plates are so thin they are translucent and under low power magnification it is possible to see that they are highly perfused with haemolymph in a similar fashion to that observed in the gills. A separate (from the one that supplies the gill) afferent haemolymph vessel which runs from the sternal sinus enters the middle of each coxal plate. It runs down to the bottom of the plate. Here it bifurcates. Each branch follows the edge of the plate but the haemolymph also seems to perfuse the whole plate. The efferent branches which have followed the edge of the plate, and the haemolymph perfusing the plates themselves reunite at the top of the plate and empty into the efferent haemolymph vessel of the limb.

Lloyd Peck is quite correct in pointing out (pers. comm.), that in what we do here (direct addition of extrabranchial area to gill area) there are likely to be errors. Essentially they are associated with, (i) the unlikely assumption that such extrabranchial gas exchange surfaces are unlikely to be perfused at the same rate as the gills and, (ii) not knowing the diffusion distances (sea water to hemolymph, i.e. cuticle and cellular thicknesses) - across both the gills and across the extrabranchial areas.

Although not formally investigated the thickness of the medial (inside facing) surface of the coxal plate seems particularly thin, as thin as the gills. Haemolymph cells are readily observable immediately beneath the cuticle of both. If it turns out they are different then we will need to incorporate a multiplier which takes into account the ratio of gill/extrabranchial thickness, when we estimate total functional gas exchange surface.

Coxal plates as extrabranchial exchange surfaces?

Cossans [2] was first to note that in *Gammarus* species, first four walking legs, were attached to enlarged coxal plates, and these were larger in *G. locusta* compared with *G. pulex*. She stated that these coxal plates functioned as ‘accessory respiratory organs, as their inner surface was covered by very thin cuticle (p.5) but did not support this with physiological measures.

Hudson and Maitland [3] presented more detailed anatomical evidence to support the idea that amphipod coxal plates were involved in extrabranchial gas exchange. They reported that the distance across the medial (i.e. inside facing) surface of coxal plates

of the semi-terrestrial talitrid amphipod *Orchestia gammarellus* was about a third of the equivalent distance across both the coxal gills and the lateral (external) surface of the coxal plates. Spicer and McMahon [4] and Spicer [5] demonstrated that gill excision or gill disease (necrosis) respectively did not alter the rate of O_2 uptake in two related talitrid amphipods. They put forward the idea that at least some of the oxygen uptake could be accounted for by gas exchange across the inside of the coxal plates.

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Supplementary materials 5.

Putative respiratory pigment in the haemolymph of *P. brevicornis*.

The absorbance peak detected at λ = 332 nm in oxygenated, but not deoxygenated, hemolymph from *Prostebbingia brevicornis in vitro* we interpret as indicating the presence of hemocyanin (Hc), a respiratory pigment that reversibly and cooperatively binds oxygen, and is used in oxygen transport in this species, for the following reasons.

(1) In all crustaceans examined to date oxygenated Hc is detected by an intense absorption peak at, or around, λ =340 nm [1,2,14]. This is close to the absorbance of the peak of λ = 332 nm detected for hemolymph from *P. brevicornis*.
(2) In all crustaceans examined to date, this oxygenated Hc peak disappears when haemolymph is deoxygenated [1,2] and the same happens in hemolymph from *P. brevicornis*.
(3) Haemocyanin that binds oxygen reversibly and cooperatively has been found in the haemolymph of most gammaridean amphipods in which it has been sought [3-10] including giant amphipods in Lake Baikal [11], (exceptions being *Paraceradocus miersi* and *Shraderia gracilis* in this present study – see below for implications of this finding). Non gammaridean groups such as the cyamid whale lice possess haemoglobin and not Hc [12] and the planktonic hyperiids possess neither [13]. This makes our interpretations in points 1 and 2 more likely for *P. brevicornis*.
(4) If we use the absorbance measures obtained at λ = 332 nm, and assume that they are associated with the presence of a haemocyanin subunit of 74 kD in mass [3] we can use the extinction coefficients for Hc produced by Nickerson and Van Holde [14] ($E^{1\%}_{cm}$ = 2.83, equating to $E^{mmol.l^{-1}}_{cm}$ = 17.26 at λ =340 nm) to calculate putative Hc concentration. For *P. brevicornis* the calculated putative Hc concentration ranges between 0.33 and 0.39 $mmol.l^{-1}$ at the upper end of the range reported for other amphipod species, 0.14 – 0.41 $mmol.l^{-1}$ [3]. This congruence strengthens the suggestion that there is Hc in the hemolymph of *P. brevicornis*.

Taken together we have presented a number of different lines of evidence that there is a Hc in the hemolymph of *P. brevicornis*. However, a number of puzzles, incongruities and alternative explanations also exist which need to be aired. They are as follows.

(1) We have not directly measured oxygen-binding by the pigment, or isolated, identified, and verified the hemocyanin molecule itself. Even if there is oxygen binding present we have no evidence that it is cooperative.
(2) While the congruence between the Hc values we might expect and what we estimate for *P. brevicornis* is good when using the extinction coefficient for the

copper peak at 340 nm, the congruence is not so good when we measure the protein peak at 280 nm and assume that it is likely to be 60-95% Hc in line with other amphipods investigated [3]. In that case the protein concentration is always lower than the value calculated based on how much Hc there seems to be. In *P. brevicornis* brought back to the UK from the Antarctic the haemolymph protein levels can be very low even though the animals are well fed and have sufficient copper in their food (JIS, pers obs). Therefore we have been unable to substantiate the claim of Hc being present in the haemolymph by (i) measuring the oxygen content of the hemolymph or (ii) replicating the disappearance of any peak at λ= 332 nm when the haemolymph is equilibrated with nitrogen gas.

Aside from oxygen transport, Hcs are also known to participate in homeostatic and physiological processes: moulting, hormone transport, osmoregulation and protein storage and as precursors of antimicrobial and antiviral peptides.

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Media summary

There is a suggestion that the potential loss of giant Antarctic species is linked with reductions in dissolved oxygen as our ocean warms in line with current climate change predictions. We suggest that while the larger species may well be more vulnerable because of oxygen limitation, the picture is a little more complex than this. Large species are not just small species writ large. Evolutionary innovation, such as the presence of respiratory pigments to enhance oxygen transport and novel gas exchange structures, in some but not all species, can to some extent offset any respiratory disadvantages of either large or small body size.